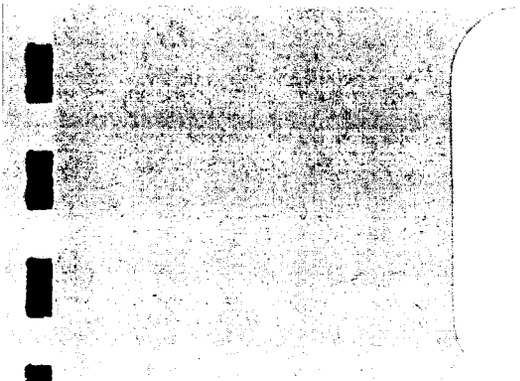

ELECTRIC BOAT GROTON, CONNECTICUT
A DIVISION OF GENERAL DYNAMICS

Photosynthetic Gas Exchange in the Closed Ecosystem for Space

Part II. Studies on the Growth of Thermophilic Chlorella 71105

NASA Contract No. NASW-95 1960-61

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FOREWORD

This paper is Part II* of the final report describing the 1960-61 studied conducted under contract NASW-95, "Photosynthetic Gas Exchanger," Phase II, issued by the National Aeronautics and Space Administration, Washington, D.C.

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* Photosynthetic Gas Exchange in the Closed Ecosystem for Space -

Part I. Pilot Photosynthetic Gas Exchange Studies, E. A. Zuraw, B.J. Weissman, R. P. Casey, V. A. Speziali, T. A. Adamson, Electric Boat Division, U411-61-131, August 1961.

Part III. Algae Screening and Mutation Studies, N. L. Richards and R. J. Benoit, Electric Boat Division, U411-61-107, August 1961.

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ABSTRACT

Bench-scale studies with Chlorella pyrenoidosa 71105 were conducted in four- and eight-liter culture vessels, with and without recycling. It was established that the algal strain could be maintained for periods up to 72 days with suplemented re-cycled medium. In a factorial series of experiments, the highest yields were obtained with a 2.0% concentration of carbon dioxide, a 0.07 dilution factor (nutrient dilution rate/culture volume), and an 8.5 ft^{-1} ratio of light surface to liquid culture volume. Optimum pH and concentrations of the constituents of the growth medium were determined in corollary test-tube studies. Other test-tube studies showed that the nutrient medium used for mass culture could be stored up to five weeks at 25°C or at refrigerator temperature (4°C) with little change in nitrogen-urea level. Algal suspensions stored at 4°C remained viable for periods of at least 12 weeks; growth the resumed after a lag period when stored suspensions were cultured at 39°C. Streptomycin was found suitable for controlling a blue-green algal contaminant.

I. Introduction

Life-support research at Electric Boat Division utilized an engineering model photosynthetic gas exchanger (reference 13). Corollary to the main studies, experiments were carried out with a bench-scale algae culture unit. Bench-scale experiments provided information concerning algal growth easily and quickly and afforded a means of comparing design and geometry with the larger photosynthetic gas exchanger. In addition to the studies carried out in a bench-scale culture vessel, support studies were also carried out in test-tubes in an illuminated water bath.

Preliminary experiments (reference 13) were conducted in 1959-60 on the effect of nutrient feed rate and re-cycled medium on algal growth. The studies reported here are a continuation of that program.

The main objectives were to study the effect of nutrient feed rate, carbon dioxide concentration, and culture volume on algal growth. Two eight-liter algae culture vessels basically similar to the four-liter units were designed, fabricated, and assembled.

A factorial experiment was carried out with the eight-liter units, and studies concerning re-cycled medium were continued in a four-liter culture vessel. In addition, test tube and flask studies were continued to obtain information concerning the composition of the growth medium.

II. Experimental Program

A. Studies with the Four-liter Culture Vessel

Two series of tests were conducted to investigate the effect of supplemented nutrient medium on algal growth. These tests were organized: 1) to determine the presence or absence of toxic materials produced by the algae over long periods of time, 2) to determine which components of the medium would first limit growth, 3) to determine if a fixed culture density could be maintained for long periods of time with a minimum amount of nutrients added to the nutrient medium. A summary of the tests conducted follows:

1. Supplemented Nutrient Medium Batch Operation
   a. Supplementing with urea
   b. Supplementing with urea and iron
   c. Supplementing with urea, iron, and calcium.

2. Supplemented Re-cycled Medium at Fixed Culture Density
   a. Supplementing with urea and iron
   b. Supplementing with urea, iron, and calcium.

B. Studies with the Eight-liter Culture Vessel

A factorial experimental study was carried out in the eight-liter algae culture units. The independent variables (nutrient feed rate or dilution rate, carbon dioxide concentration, and culture volume) were selected on the basis of earlier work.

The dilution rates studied correspond to dilution factors of 0.04, 0.07, and 0.1. Dilution factor is the dilution rate divided by the volume of the culture suspension; it is the reciprocal of the retention time as used in chemical engineering. Zuraw et al (reference 13) found that the ratio of illuminated area to the culture volume had a significant effect on growth of Chlorella. Since the area of light surface exposed was fixed by the design of the units at 1.5 ft^2, the only way to study this variable was to use different culture volumes. The two volumes used were five liters and eight liters. Therefore, the ratios of lighted surface to culture volume were 8.5 and 5.3 ft^2/ft^3 respectively. The dependent variables measured were equilibrium culture density and algae production or yield. Oxygen production is known to be proportional to growth. Consequently, the gas exchange capacity can be estimated from algal production.

Table I gives a summary of the independent, fixed, and dependent variables studied in the eight-liter culture vessels. Thus, two factors at three levels, and one factor at two levels give eighteen possible treatment combinations, which replicated once, give a total of thirty-six experiments.

TABLE I

Outline of the Factorial Experiment

Independent Variables	Dependent Variables	Fixed Factors	Levels
Dilution Factor			0.04
			0.07
			0.10
Carbon Dioxide Concentration			0.03%
			1.0%
			2.0%
Lighted Surface/ Volume			5.3
			8.5
	Algae Production Equilibrium Density		
		Temperature	39°C
		Standard Medium	See Appendix A
		Light Intensity	6000 ft. c.*
			(240 volts)

*As measured at inner surface of the culture.

C. Flask and Test Tube Studies

Studies conducted in test tubes and flasks concerned the following:

1. Storage stability of medium
2. Storage stability of Chlorella suspensions
3. Effect of Streptomycin and Phygon*, on Chlorella 71105 and a blue-green contaminant
4. Studies on the composition of medium on growth of algae

III. Materials and Methods

All experimentation was conducted using Sorokin and Myer's thermophilic, Chlorella pyrenoidosa 7-11-05 (reference11), a stock culture of

*Phygon XL is a chlorinated napthoquinone fungicide, produced by the Naugatuck Chemical Division of U.S. Rubber Co.

which is maintained in our laboratories. For convenience, we have re-designated the strain Chlorella 71105.

A. Studies with the Four-liter Culture Vessel

This unit is identical to the one operated in previous studies reported by Zuraw, et al (reference 13). A detailed description and a photograph of the unit appear on pages 50 and 51 of that report.

1. Study of Supplemented Nutrient Medium

The studies were essentially of two types. In the first series of experiments, the unit was inoculated, and the various supplementary nutrients were added to the culture whenever the packed cell volume indicated no further increase in population or whenever a urea-nitrogen determination indicated low nitrogen status.

In the second series of experiments, the unit was inoculated, and the culture density was maintained at approximately 1% packed cell volume (PCV) by daily harvests from the unit. The algae cells were removed from the daily harvests by centrifugation and the effluent medium returned to the unit with appropriate mineral supplements.

In both series of experiments, carbon dioxide concentration, temperature, and light intensity were kept constant. Carbon dioxide concentration was maintained between 1 and 2%. The power to the lamp was set at 240 volts, as indicated by a voltmeter. Foaming was controlled by using Dow-Corning Antifoam B. Volume-loss due to evaporation was made up with deionized water.

B. Studies with the Eight-liter Culture Vessel

The eight-liter culture vessel is shown in Figure 1. The design is similar to the original four-liter model. The larger unit has a Pyrex glass cooling-water jacket rather than plastic. The diameter of the glass annulus is 5.75 in. O.D. as opposed to a 5.0-inch plastic annulus

of the four-liter unit, resulting in a larger area of light surface exposed, 1.5 ft^2 compared with 0.9 ft^2 in the smaller model. The new units have "O" ring-seals about the glass tubes as opposed to a large rubber dam in the older model. The rectangular algae chamber with a hemi-cylindrical bottom is made of a clear acrylic plastic. The vessel is 12-inches long, 8-inches wide, and 12-inches deep. The 1.5 KW Quartz-line lamp is housed in a 2-inch glass tube centered in the cooling water jacket. The cooling system, light intensity control, nutrient feed, algae temperature control, and culture turbulence and circulation were achieved in the same manner as that described for the studies conducted in the four-liter units reported in reference 13.

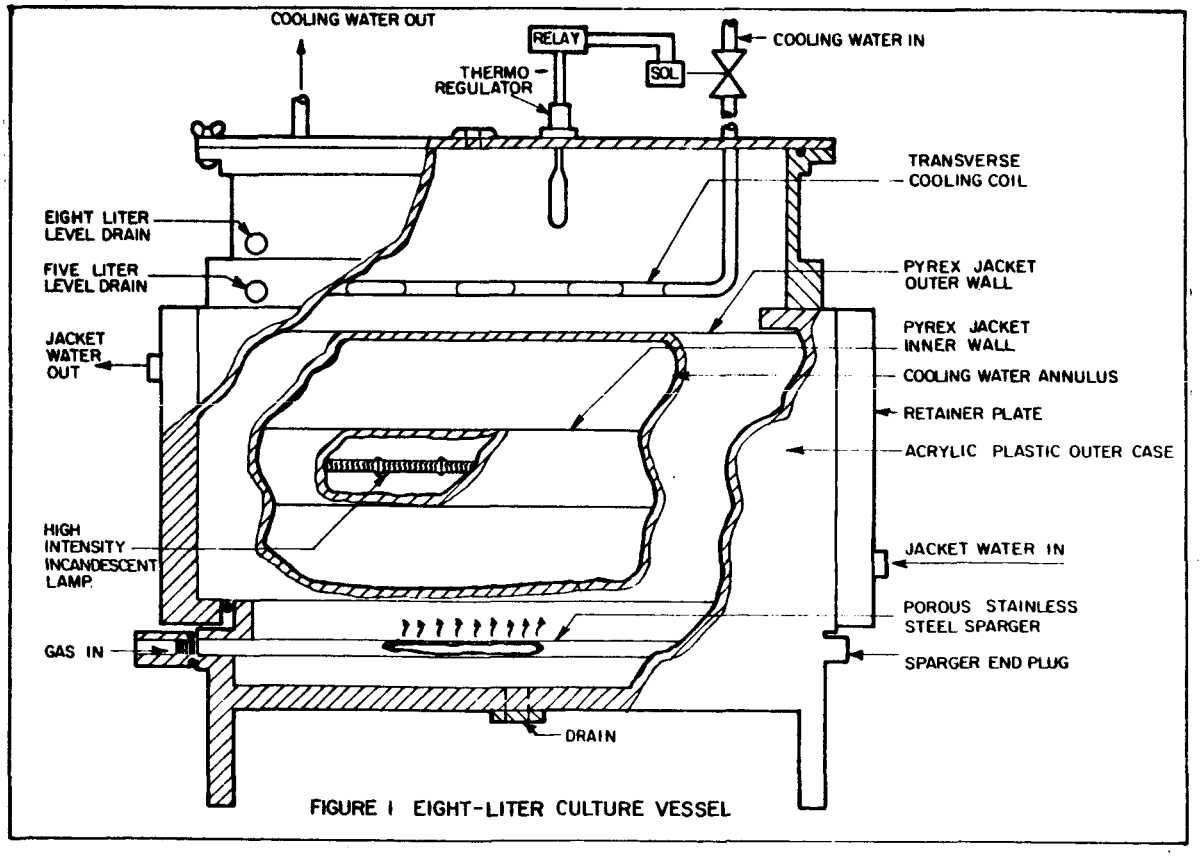

FIGURE 1 EIGHT-LITER CULTURE VESSEL

1. Operation of the Eight-liter Units

The culture vessels were inoculated with a suspension of algal cells. Nutrient was fed at pre-selected rates by means of calibrated Lapp metering pumps. The volume of the cultures was controlled by drain lines at fixed levels. Carbon dioxide and air were mixed in equipment similar to that described on page 247 of the 1953 Carnegie Institute Report (reference 1). The carbon dioxide concentration was checked periodically with a Haldane-Guthrie Gas analysis apparatus. Cultures were allowed to reach a steady-state or equilibrium density. Other environmental factors such as temperature, light intensity, and composition of the medium were kept constant. The temperature was maintained at $38.5^{\circ}\pm1^{\circ}C$. The light intensity was measured at the end of the experimental program with a Weston Wollensak Fastax Exposure Meter, Model 755, and a value of 16,000 foot candles was obtained. In this instance, however, the glass water jacket was cleaned.

Appendix C shows the light intensity values obtained with a G.E. photographic light meter and a Weston Foot-candle Meter, Model 614, under actual operating conditions. Values of approximately 6000 ft.c. were obtained in this instance. Over an extended period of time, a slightly opaque deposit forms in the water jacket. (In future studies cooling water will be recirculated to an external heat exchanger.)

Standard laboratory medium as outlined in Appendix A was used throughout the studies.

Data concerning pH, carbon dioxide concentration, and PCV were obtained every 3 hours during the day. When steady-state conditions were obtained, dry weight and PCV determinations were made using the technique described in Appendix B.

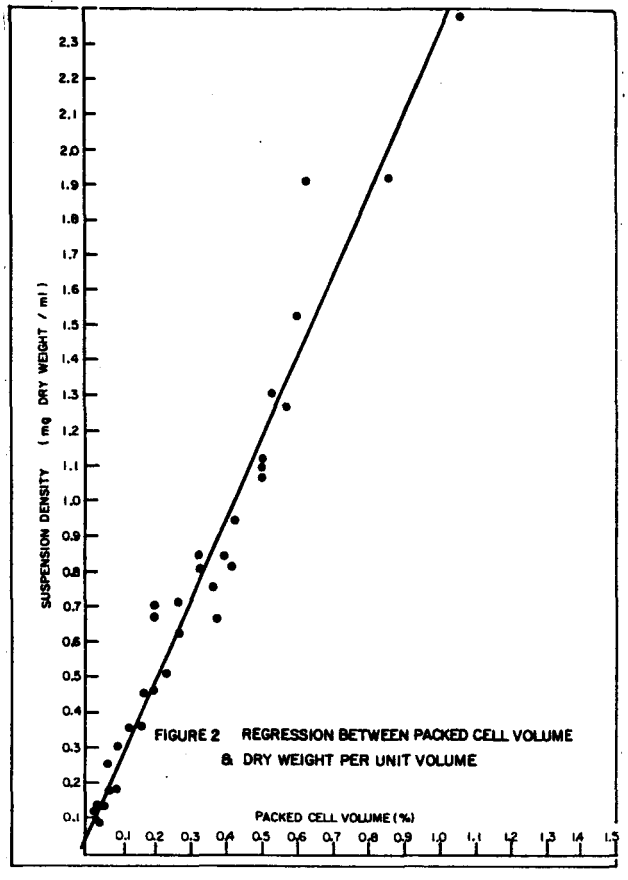

FIGURE 2 REGRESSION BETWEEN PACKED CELL VOLUME & DRY WEIGHT PER UNIT VOLUME

Growth of Chlorella and similar microalgae has been measured at Electric Boat Division and in other laboratories in a variety of ways, including optical density of cell suspensions, direct cell counts, packed cell volume obtained from centrifuging a measured volume of suspension, and/or by the weight of the dried residue from a measured volume of centrifuged and washed cells. Both packed-cell volume and dry-weight determinations were carried out in the present study. Data for these two measures are given in Figure 2; the correlation between the two measures is +0.98. The equation of the line of best fit is 2.304(PVC)+0.0237 = Dry weight of algae in mg/ml.

C. Flask and Test Tube Studies

These experiments were carried out in 650 ml or in 100 ml test tubes in a lighted aquarium tank similar to the unit used in studies conducted by Zuraw et al (reference 13).

The temperature of the water in the aquarium water-bath was maintained at $38.5^{\circ}\pm1^{\circ}C$, with a thermostat, relay, and immersion heater. The bath is equipped with a stirrer and two air-lift filters. A gas mixture of air and carbon dioxide was distributed through a manifold equipped with needle valves to a glass gassing tube in each test tube. The 100 ml test tubes were placed in a plastic rack immersed in the water-bath. The large volume test tubes were placed directly into the water-bath thru cut-outs on the cover of the aquarium. Fixtures containing three 40-W Power-groove* fluorescent lamps flanked the aquarium, providing light at approximately 2000 foot candles.

*Registered trademark of General Electric Company

The flask studies were carried out in 500 ml Erlenmeyer flasks on a screen over an inverted fluorescent light fixture, providing a light of approximately 2000 foot candles, as measured with a Weston Wollensak Fastax Exposure Meter, Model 755.

Temperature in flask tests was not controlled but remained fairly constant at 37° to 39°C. Air was passed over the lamps to aid in maintaining this flask temperature. The gas mixture was supplied in the same manner as in the aquarium unit.

In flask and test tube studies, algae growth was followed by one of three methods:

1. Packed cell volume.
2. Optical density determined with a Fisher Electro-photometer at 550 millimicrons.
3. Direct cell counts using a bright-line hemacytometer.

Urea nitrogen determinations were made using the urease-aeration method of Van Slyke and Cullen (reference 8).

IV. Results and Discussion

A. Studies with the Four-liter Culture Vessel

In the standard laboratory medium, $MgSO_4$, KH_2PO_4, and NaCl are considered to be present far in excess of the daily requirements for even dense Chlorella cultures. Urea, iron and calcium are considered supplementary and are routinely added at daily intervals in the dense cultures.

1. Supplemented Nutrient Medium Studies, Batch Operation

a. Supplementing with urea only. (Figure 3, Curve A.)

A maximum culture density of 2.5% PCV was attained by supplementation with urea. The culture grew steadily for four days without supplements, reaching a density of 1.20% PCV before levelling off. Urea (380 mg/L) was added to the culture and growth resumed, reaching a density of 2.3%

by the 7th day. An additional amount of urea (380 mg/L) was added to the culture and a maximum density of 2.5% was reached by the ninth day. The addition of urea alone after the ninth day had no effect on the algae culture and the culture density gradually declined.

b. Supplementing with urea and iron. (Figure 3, Curve B.)

Urea (380 mg/L) and iron (0.56 mg/L) were added daily to an algae culture and the growth measured daily. The growth continued steadily for 18 days at which time the culture density was 5.0%. The further addition of urea and iron to the culture resulted in no increase in growth.

c. Supplementing with urea, iron and calcium. (Figure 3, Curve C.)

Urea (380 mg/L), iron (0.56 mg/L) and calcium (8 mg/L) were added daily to an algae culture and growth measured daily. Growth steadily increased for 14 days to a density of 4.8%. Further addition of these supplements to the culture did not cause an increased density, but rather the culture leveled off at approximately 5.0%. This density was maintained for 30 days, at which time the culture was discontinued.

It should be noted that in all of the above experiments the initial culture media were made with tap water as were the supplement stock solutions.

Figure 3 shows the results of the three experiments conducted. It is apparent that iron as a supplement is as important as urea. With the addition of calcium as a supplement no significant increase in density was noted; however, the density of 5% (PCV) was maintained for a longer period of time. It is assumed that at this high density of 5% (PCV) the algae cells were light-limited. It is apparent that no autotoxic materials were produced by the algae culture.

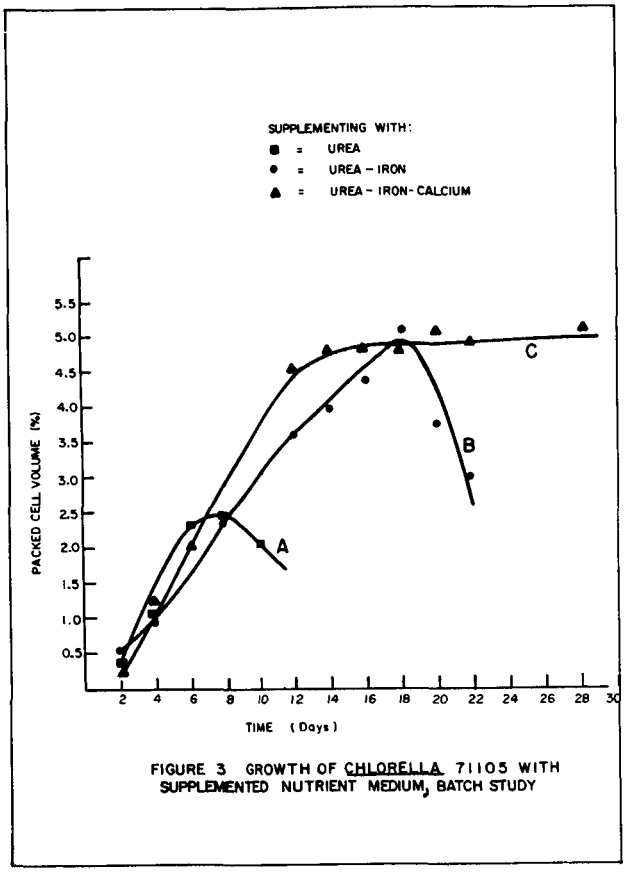

FIGURE 3 GROWTH OF CHLORELLA 7110S WITH SUPPLEMENTED NUTRIENT MEDIUM, BATCH STUDY

In all three experiments, the pH of the culture medium gradually increased to 7.5 from an initial unadjusted pH of 4.6. At the high pH, a precipitate was observed in the culture. A portion of this material was collected, washed, and dried. A semi-quantitative spectroscopic analysis was made by the Spectroscopy Laboratory of the Technical Service Division. Table II shows these results.

The material is apparently magnesium phosphate, which exists in a number of hydrated forms. The Debye powder x-ray diffraction pattern indicates the presence of $MgHPO_4.3H_2O$; $MgNH_4PO_4.6H_2O$ and $Mg_3(PO_4)_2.4H_2O$ in the precipitate.

TABLE II

Semi-quantitative Spectroscopic Analysis of a Precipitate formed in Re-cycled Medium

% Composition					
Greater than 10.0	1 to 10	0.1 to 1.0	0.01 to 0.1	0.001 to 0.01	Less than 0.0001
Phosphorus	Magnesium	Silicon	Calcium Iron	Barium Copper Sodium Titanium	Aluminum Bismuth Boron Chromium Lead Manganese Tin

The presence of a high bacterial population was noted during all of these experiments with supplemented medium, sometimes as high as 26 X 10^7 per ml; however, this did not seem to affect the growth of algae. A suspension density of 5.0% was recorded in this instance.

2. Supplemented Re-cycled Medium at Fixed Culture Density

Four experiments were completed of 19, 71, 22 and 36 days duration. Whenever mechanical difficulties with the culture vessel occurred, the culture was harvested and refrigerated until the unit was repaired. The culture was then replaced in the unit and the experiment continued. Figure 4 shows the results of a preliminary experiment with culture density adjusted daily. On the seventh day an accident occurred; the culture was drawn off and placed in the refrigerator. Seven days later, after the unit had been repaired, the culture was restored to the vessel. Growth at a high rate commenced after a short lag period.

Figures 5 through 7 show the results of later experiments. In run #2 (Figure 5) an interesting phenomenon occurred on the 33rd day. A gradual decline in culture density was evident and yet, with the addition of the major elements of the nutrient to the culture, no increase in density was apparent. On the 37th day, 16 ml micronutrients (Arnon's A-5 solution) were added to the culture resulting in a marked increase in culture density. During the same experiment on the sixtieth day a decrease in

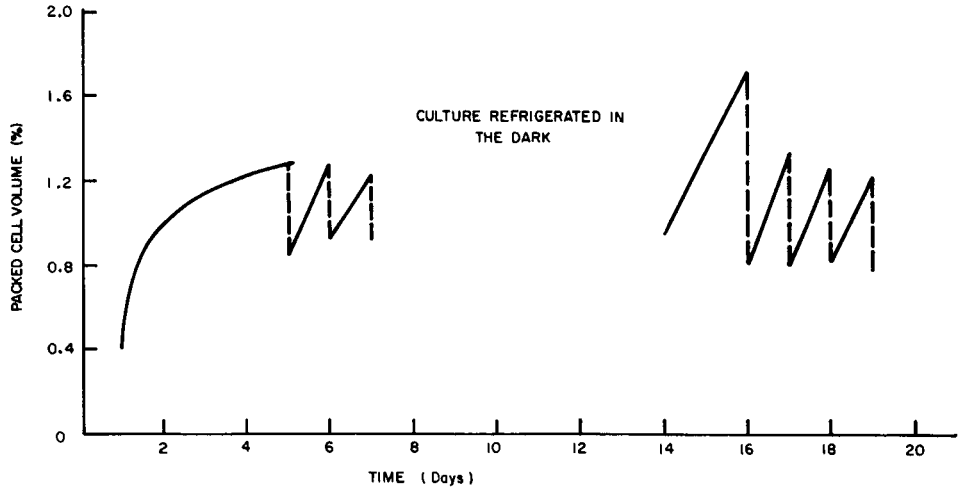

FIGURE 4 EFFECT OF RECYCLED MEDIUM SUPPLEMENTING DAILY WITH UREA & IRON, MAINTAINING DENSITY BY HARVESTING DAILY

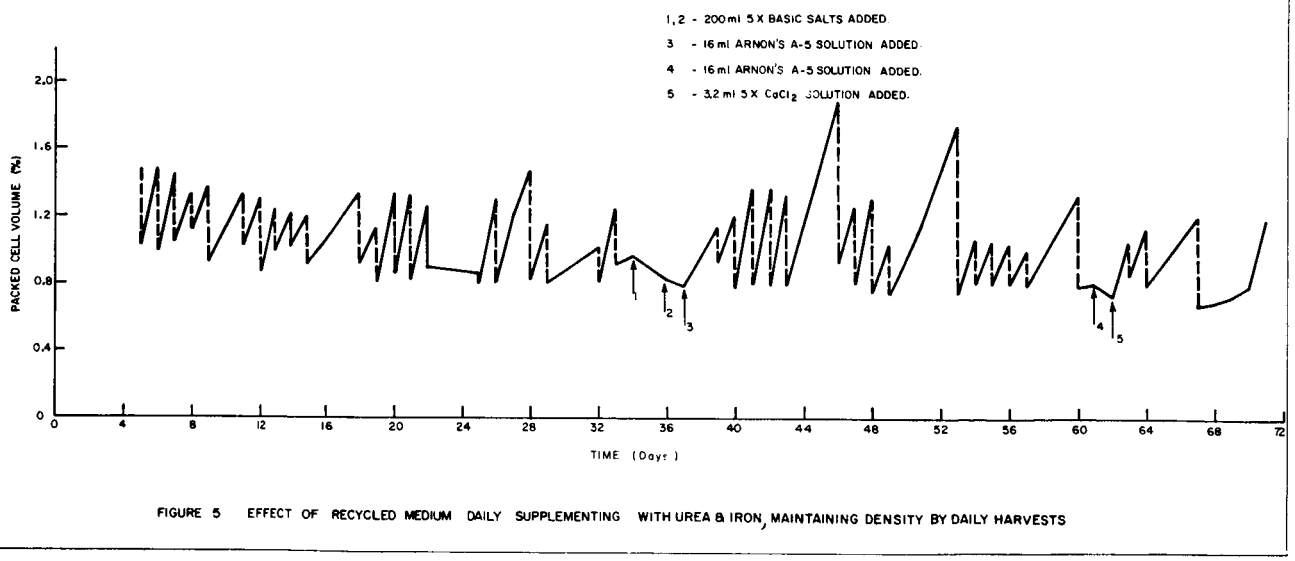

FIGURE 5 EFFECT OF RECYCLED MEDIUM DAILY SUPPLEMENTING WITH UREA & IRON, MAINTAINING DENSITY BY DAILY HARVESTS

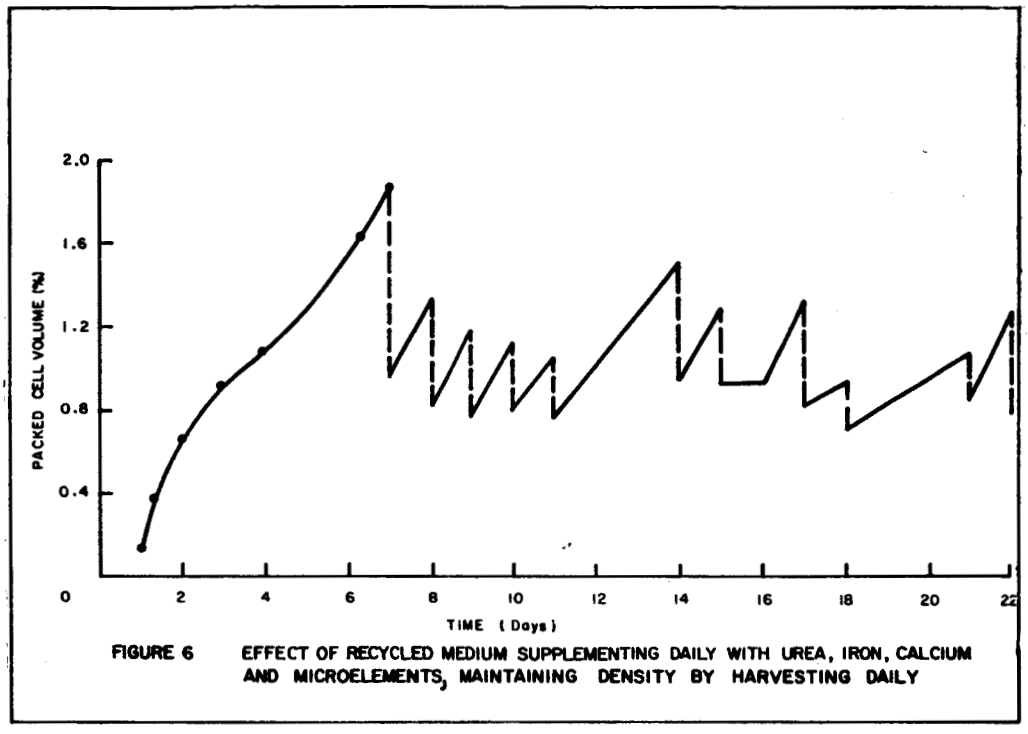

FIGURE 6 EFFECT OF RECYCLED MEDIUM SUPPLEMENTING DAILY WITH UREA, IRON, CALCIUM AND MICROELEMENTS, MAINTAINING DENSITY BY HARVESTING DAILY

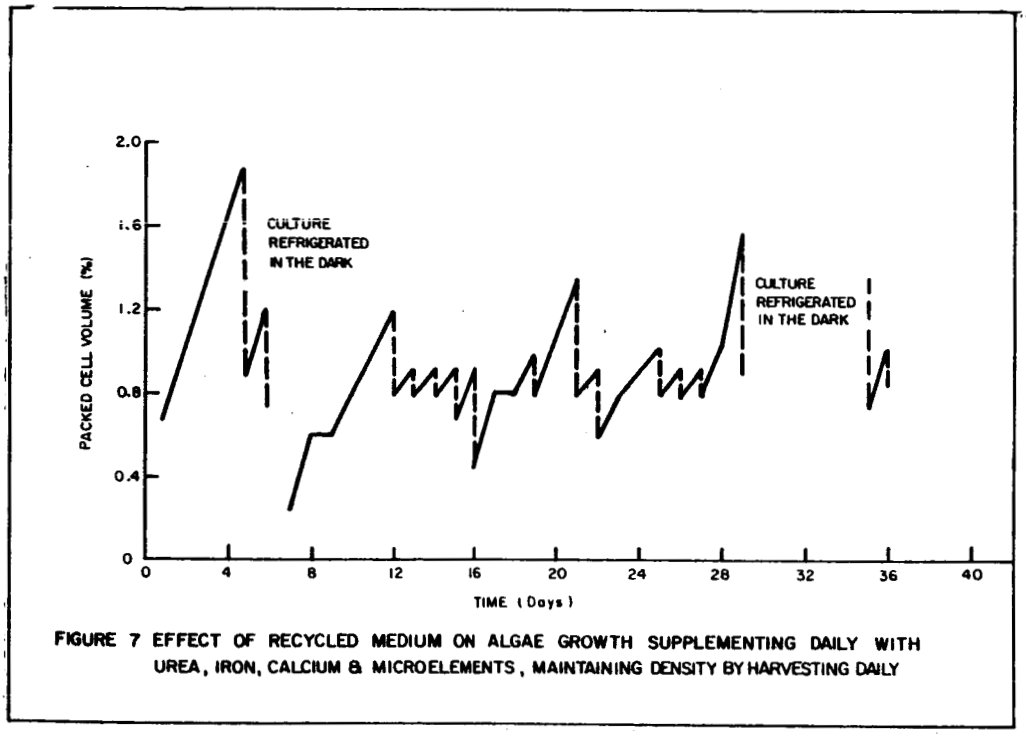

FIGURE 7 EFFECT OF RECYCLED MEDIUM ON ALGAE GROWTH SUPPLEMENTING DAILY WITH UREA, IRON, CALCIUM & MICROELEMENTS, MAINTAINING DENSITY BY HARVESTING DAILY

density was observed, but the addition of micronutrients did not influence the culture density. However, when calcium chloride was added to the culture an increase in density was evident. Figures 6 and 7 show two runs of 22 and 36 days duration, supplemented daily with urea, iron, calcium chloride, and the microelements. These experiments verify results of the previous studies. From these experiments, it is apparent that minimal amounts of autotoxic products are produced by Chlorella 71105, and that rather dense cultures (approximately 1% density) can be maintained for long periods of time with a supplemented re-cycled medium.

B. The Factorial Experiment

The factorial experiment was performed with dilution factor, carbon dioxide, and culture volume as independent variables. Levels of dilution factor used were 0.04, 0.07, 0.1; dilution factor is the ratio of nutrient feed rate (ml/hr) to culture volume (ml). Levels of carbon dioxide used were 0.03%, 1.0%, and 2%. The two volumes used were five liters and eight liters. The surface of the lamp water jacket has an area of 1.5 ft^2; therefore the ratios of lighted surface to culture volume were 8.5 and 5.3. The dependent variables evaluated were equilibrium density and algae production. Methods of calculation are outlined in Appendix B. The data for all equilibrium runs appear in Table III. The results given are averages of duplicates. Factors held constant are listed below:

1. Nutrient concentration - Standard Laboratory Medium
2. Temperature - $38.5^{\circ} \pm 5^{\circ}C$.
3. Light power supply - 240 volts. (Equivalent to about 6000 ft.c. at the surface of the lamp jacket)

Table IV gives the data for the effects of the independent variables on equilibrium density. An analysis of variance (reference 3 and 7) was performed on the data. The analysis determines which independent variable significantly affected the dependent variables. Table V

TABLE III

Results of Factorial Experiments with the Eight-liter Culture Units
(Results are averages of duplicate runs)

Experiment No.	Carbon Dioxide Conc. %	Light Surface Volume Ratio ft^2/ft^3	Dilution Factor	Packed Cell Volume %	Equilibrium Density mg/ml	Algae Produced gm/hr	Specific Yield gms/hr	Algae Produced lbs/hr	Doubling time hrs	Flow ml/hr
1	1.0	5.3	0.07	0.31	0.75	0.417	$52.5X10^{-3}$	$9.0X10^{-4}$	9.9	560
2	1.0	5.3	0.10	0.05	0.15	0.128	14.7	2.5	6.9	800
3	1.0	5.3	0.04	0.38	0.81	0.259	32.4	5.7	17.3	320
4	1.0	8.5	0.07	0.50	2.00	0.383	76.7	8.3	9.9	350
5	1.0	8.5	0.10	0.20	0.58	0.291	58.2	6.4	6.9	500
6	1.0	8.5	0.04	0.59	1.40	0.279	55.9	6.2	17.3	200
7	2.0	5.3	0.07	0.27	0.67	0.374	46.8	8.2	9.9	560
8	2.0	5.3	0.10	0.19	0.56	0.450	56.3	9.9	6.9	800
9	2.0	5.3	0.04	0.44	0.92	0.295	36.9	6.5	17.3	320
10	2.0	8.5	0.07	0.75	1.91	0.670	133.7	14.7	9.9	350
11	2.0	8.5	0.10	0.42	0.89	0.445	89.0	9.8	6.9	500
12	2.0	8.5	0.04	1.06	2.50	0.497	99.5	10.8	17.3	200
13	0.03	5.3	0.07	0.06	0.19	0.103	13.5	2.5	9.9	560
14	0.03	5.3	0.10	0.09	0.21	0.165	20.6	3.6	6.9	800
15	0.03	5.3	0.04	0.13	0.28	0.088	11.0	1.4	17.3	320
16	0.03	8.5	0.07	0.85	0.24	0.081	16.5	1.8	9.9	350
17	0.03	8.5	0.10	0.04	0.11	0.056	11.3	1.2	6.9	500
18	0.03	8.5	0.04	0.25	0.50	0.101	20.1	2.4	17.3	200

TABLE IV

Equilibrium Density (mg/ml)

Light Surface/Liquid Volume (ft^2/ft^3)

Dilution Factor (hr^{-1})	5.3 Carbon Dioxide Concentration %			8.5 Carbon Dioxide Concentration %		
	0.03	1.0	2.0	0.03	1.0	2.0
0.04	0.365 (15)*	0.810 (3)	1.095 (9)	0.660 (18)	1.527 (6)	2.610 (12)
	0.185	0.810	0.750	0.355	1.270	2.365
0.07	0.255 (13)	0.845 (1)	0.622 (7)	0.300 (16)	1.120 (4)	1.920 (10)
	0.132	0.645	0.715	0.173	1.070	1.910
0.10	0.260 (14)	0.175 (2)	0.672 (8)	0.139 (17)	0.702 (5)	0.840 (11)
	0.152	0.120	0.455	0.087	0.465	0.940

*Refers to experiment number in Table III

TABLE V

Analyses of Variance (Equilibrium Density)

Source of Variation			Degrees of Freedom	Variation Estimation	Variation Ratio (Pooled)	Statistical "F" Value 95% Level	Significant
(A) CO_2 Level			2	2.544	14.13	3.44	S
Order	Effect	Balance					
Linear	5.086	0.002	(1)				S
Quad-ratic	0.002	0.000	(1)				NS
(B) LS/V			(1)	2.873	15.96	4.30	S
Order	Effect	Balance	(1)				
Linear	4.563	-1.69					
(C) Dilution Factor			2	1.030	5.72	3.44	S
Order	Effect	Balance					
Linear	2.050	0.009	(1)				S
Quad-ratic	0.009	0.000	(1)				NS
AB			2	0.574	3.19	3.55	NS
AC			4	0.281	1.56	2.82	NS
BC			2	0.147	.81	3.44	NS
Residual			22	0.180	-	-	NS
Totals			35	-	-	-	-

TABLE VI

Yield of Algae (gm/hr) x 10^{-3}

	Light Surface/Liquid Volume Ratio (ft^2/ft^3)					
	5.3 Carbon Dioxide Concentration %			8.5 Carbon Dioxide Concentration %		
Dilution Factor (hr^{-1})	0.03	1.0	2.0	0.03	1.0	2.0
0.04	14.6 (15)*	32.4 (3)	43.8 (9)	26.3 (18)	61.1 (6)	104.4 (12)
	7.4	32.4	30.0	14.2	50.8	94.6
0.07	17.8 (13)	59.1 (1)	43.5 (7)	21.0 (16)	78.4	134.4 (10)
	9.2	45.2	50.0	11.7	74.9	133.8
0.10	26.0 (14)	17.5 (2)	67.0 (8)	8.7 (17)	70.0	84.0 (11)
	15.2	12.0	95.5	13.9	46.5	94.0

* Refers to experiment number in Table III

showsthe analysis of variance for equilibrium culture density. It confirms with 95% confidence that within the range used, a change of 1% carbon dioxide has a significant effect on equilibrium density: increased CO_2 results in a linear increase in equilibrium density. It shows further that a quadratic expression for this relationship would not yield a significant improvement over a straight line. The two levels of light surface-to-volume ratio gave significantly different equilibrium densities. The changes in dilution rate also resulted in significant changes in equilibrium density. The error mean square was very small as was the three factor interaction. Therefore these were pooled for "F" tests. None of the two factor interactions were statistically significant in the ranges of levels used, i.e., the factors behaved independently. The coefficient of correlation between corresponding observation of two sets of runs is +0.98. This demonstrates that the degree of reliability for these data is quite satisfactory, and the experiments are reproducible.

Table VI gives the data for the effects of the independent variables on algae production (specific yield). Analysis of variance for algae production (specific yield) is given in Table VII. As indicated in the table, carbon dioxide concentration, culture volume, and dilution factor significantly affected yield. A curvilinear relationship between dilution factor and yield is apparent from Figure 9. All of the two factor interactions were statistically significant in the ranges of levels used. The coefficient of correlation between corresponding observation of duplicate runs is greater than 0.9.

All the relationships between the independent variables and equilibrium densities and yields are shown in Figures 8 through 11. Figures 8 and 10 represent the effect of carbon dioxide concentration on equilibrium density and algae production. Figures 9 and 11 show the effect of dilution rate on these responses. In all cases best results were obtained at the higher light surface-to-liquid volume ratio (5 liters versus 8 liters culture volume). The highest carbon dioxide level studied also gave the highest yield. A dilution factor of 0.07 gave

TABLE VII

Analysis of Variance (Specific Yield of Algae)

Source of Variation			Degrees of Freedom	Variation Estimation	Significant
A. CO_2 Level			2	12965.903	S
Order	Effect	Balance			
Linear	25931.800	0.006			S
Quad	0.003	0.003			NS
B. LS/V			1	7061.604	S
Order	Effect	Balance			
Linear	21259.219	-14197.615			
C. Dilution Factor			2	637.203	S
Order	Effect	Balance			
Linear	60.802	1213.604			
Quad	1213.602	0.002			S
AB			2	2012.333	S
AC			4	366.908	S
BC			2	473.630	S
ABC			4	700.880	S
Within Cells			18	73.339	
Totals			35		

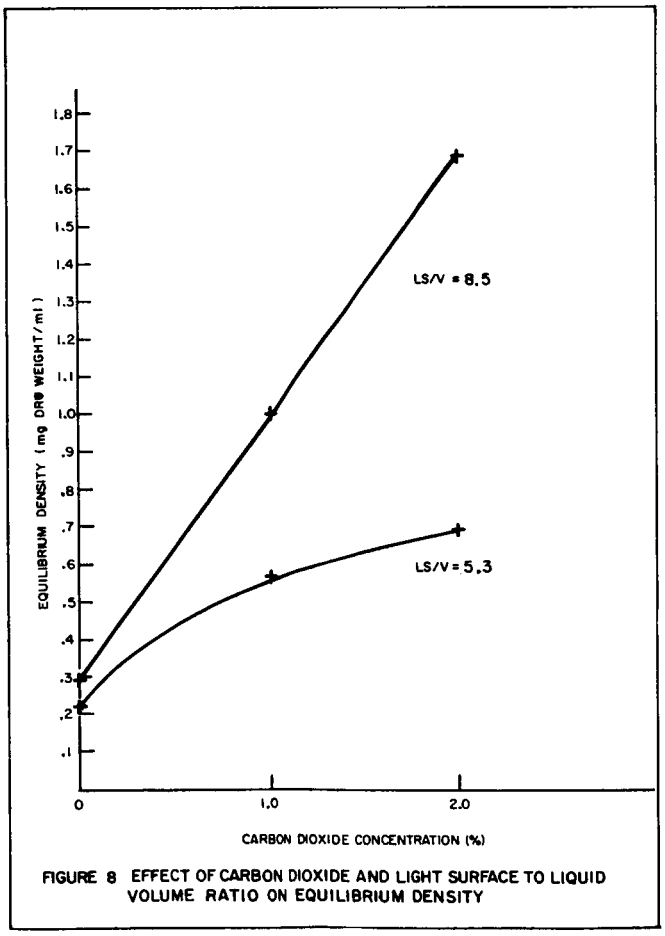

FIGURE 8 EFFECT OF CARBON DIOXIDE AND LIGHT SURFACE TO LIQUID VOLUME RATIO ON EQUILIBRIUM DENSITY

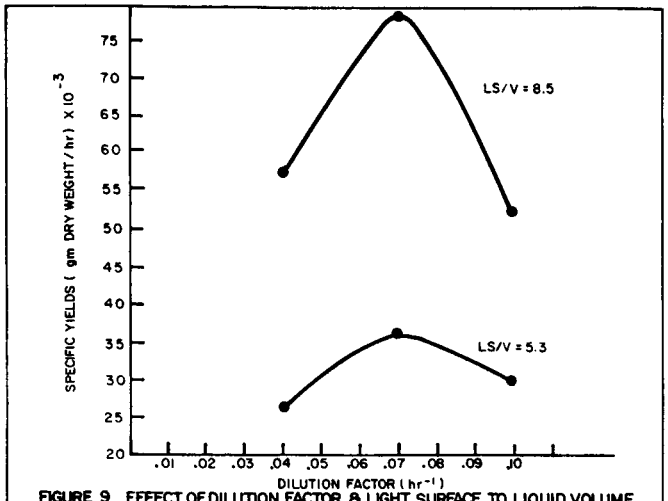

FIGURE 9 EFFECT OF DILUTION FACTOR & LIGHT SURFACE TO LIQUID VOLUME RATIO ON SPECIFIC YIELD

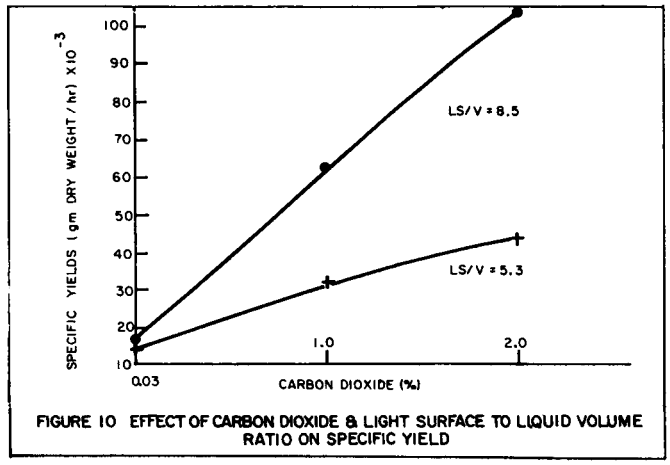

FIGURE 10 EFFECT OF CARBON DIOXIDE & LIGHT SURFACE TO LIQUID VOLUME RATIO ON SPECIFIC YIELD

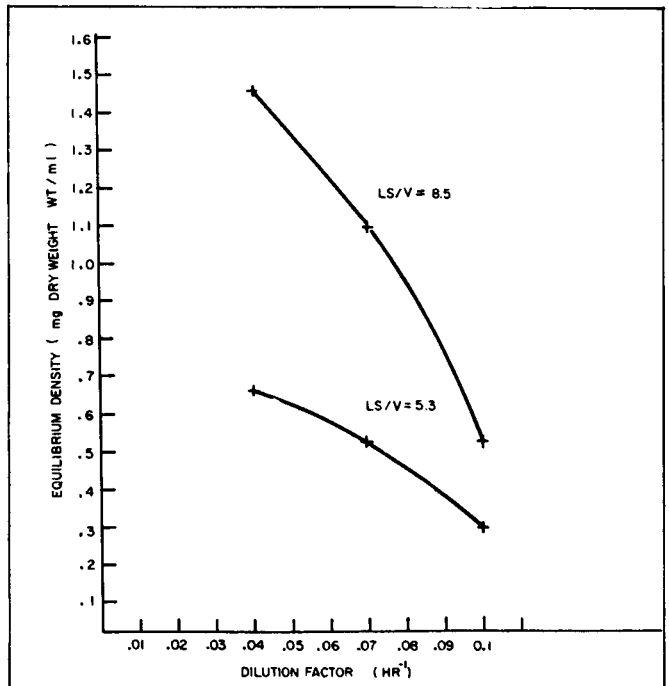

FIGURE 11 EFFECT OF DILUTION FACTOR AND LIGHT SURFACE TO LIQUID VOLUME RATIO ON EQUILIBRIUM DENSITY

the best yield; however, the highest equilibrium density was obtained at a lower dilution factor (0.04). This confirms results of Zuraw et al (1960), reference 13. The optimum level of carbon dioxide concentration is obviously higher than 2.0% according to Figures 8 and 10, but Gaucher et al (1960), reference 5, obtained better growth at 0.5% CO_2 than at 3.0% or 5.5%. The optimum apparently lies above 2.0% but below 3%. (The light intensities used by Gaucher et al (1960) were about the same as used in our experiments.)

The optimum level of CO_2 and the possible interaction between CO_2 and light intensity should be investigated further.

Figure 12 gives data for all experiments conducted with the eight-liter units. Specific yields have been plotted against equilibrium densities. The dimensions of these parameters are such that three straight lines should be obtained, each corresponding to a particular dilution factor. From the figure, it is apparent that best yields and highest equilibrium densities occur at the higher light surface to culture volume ratio. It should be noted also that yields group themselves into three classes corresponding in a general way to the three levels of carbon dioxide used. It is apparent that the higher dilution factor resulted in high yield at low equilibrium density. The lowest dilution factor gave the highest densities, but maximum yield occurred at the intermediate dilution factor, confirming the results of Zuraw et al (reference 13).

C. Flask and Test Tube Studies

1. Storage Stability of Medium

In programs involving mass cultures of algae, it becomes impractical to make up large volumes of medium. Often the medium will be stored for long periods of time before use. A simple storage test was conducted in order to determine the quality of medium upon standing for long periods of time at room temperature and at refrigerator temperature. Standard medium was prepared and stored under the two conditions of

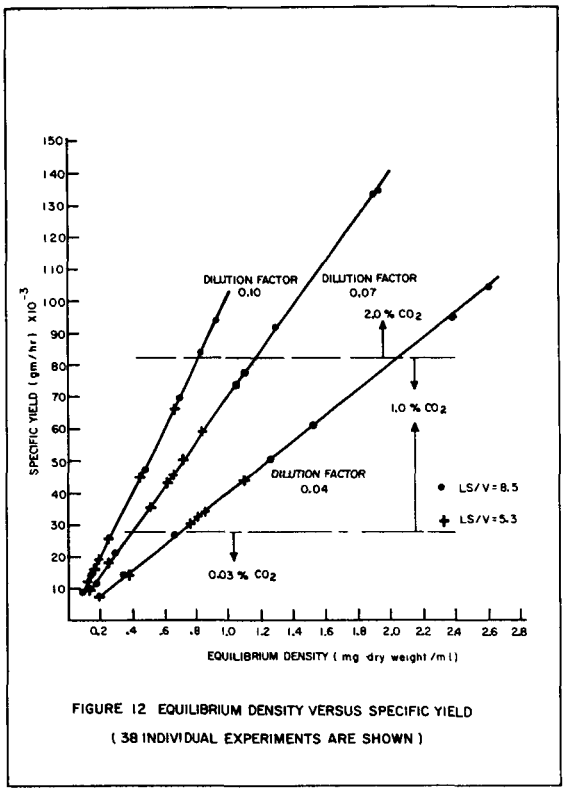

FIGURE 12 EQUILIBRIUM DENSITY VERSUS SPECIFIC YIELD
(38 INDIVIDUAL EXPERIMENTS ARE SHOWN)

temperature. At one week intervals, urea nitrogen, pH, and a bacterial count were determined. The results indicate that the medium can be stored at room temperature and at refrigerated temperature up to five weeks with little change in urea, nitrogen, and pH values. The total bacterial count on refrigerated medium was virtually zero even after prolonged storage, while the medium held at room temperature contained a small number of bacteria. However, two samples stored at room temperature showed evidence of contamination with a filamentous blue-green alga within 3 weeks. This event is obviously much more likely to occur in a busy laboratory where many people are working with a variety of cultures than in a space vehicle.

2. Storage Stability of Chlorella Suspensions

It will undoubtedly be necessary in any algal gas exchange operation to store stock cultures as standby for the main cultures. With this in mind, a study was conducted in order to determine if algae stored as a dilute cell suspension or a concentrated paste remain viable after prolonged periods. Eosin-y stain was used as a presumptive test for viability. Eosin-y stains dead cells but not living tissue; it has been

used, for example, to test for viability in protozoan cysts (reference 2). Preliminary tests were conducted to determine if this method were applicable to Chlorella.

TABLE VIII

Viability Tests with Chlorella 71105

	Algae cells treated with 1% Eosin-y Stain		Algae cells treated with 1% Eosin-y Stain, then plated	Algae cells treated with 1% Eosin-y, washed, and plated
	Stained %	Not Stained %		
Treated 90°C 1 hour (non-viable)	98.8	0.2	No growth	No growth
Not heat treated (viable)	0.03	99.97	No growth	Good growth

Table VIII show the results of this test. Algal cells heated at a temperature of 90°C for one hour were considered to be non-viable, whereas untreated cells were considered viable. These suspensions were treated with the stain and the cells counted on a bright line hemacytometer. Microscopic examination of cells treated with the stain indicated that this method is applicable to Chlorella. It is interesting to note from Table VIII that viable algae cells treated with the stain and plated out on a standard medium containing agar did not grow. However, growth did occur when the stain was washed from the cells and the cells plated out.

In the storage tests, algae cells were harvested as a dilute cell suspension (0.8% PCV) and as a concentrated suspension (10.4%). These cells were stored at 4°C and periodically an aliquot of the cells were stained and counted to determine the percentages of stained and unstained cells. After 12 weeks,

99.5% of the cells stored as a dilute suspension still did not take the stain, and 92.3% of the cells stored as a concentrated suspension did not take the stain, showing that the majority of cells present were presumptively still viable.

When these stored cells were inoculated into fresh medium in test tubes and incubated in the light, a definite lag period was observed. Active cells exhibited a characteristic lag period of about 17 hours before visible growth occurred. The cells stored as a dilute suspension showed visible growth only after 40 hours. Those stored as a concentrated suspension showed visible growth after 60 hours. This phenomenon, if confirmed, would suggest that active stock cultures should be maintained in support of large cultures used for gas exchange in spacecraft. Frequently sub-cultured, active stock cultures are routinely maintained in our laboratories.

3. Effect of Phygon XL, and Streptomycin on Chlorella 71105 and a Blue-green Algal Contaminant

During the experiments conducted with the eight-liter culture vessels, Chlorella cultures became contaminated on occasion with a filamentous blue-green alga with small moniliform cells. Preliminary tests were conducted in order to control or eliminate them. The plastic material used in construction of the culture vessels made it impossible to sterilize the units by autoclave. Therefore, methods of chemical control were sought.

These tests were carried out in flasks illuminated from below with fluorescent lamps. The effect of chemicals on the algae was noted by visual observation.

Phygon XL, (see Footnote page 5) has been shown to

be selectively toxic to blue-green alga (reference 4). Certain species were completely inhibited or killed by 5 µgm/L. Green algae were far less sensitive. A sample of Phygon XL (50% active) was kindly provided by Dr. Douglas Tate.

The material was tested at 10, 50, 500 and 1000 µgm/liter (100% active basis) against the blue-green and against Chlorella 71105. No marked inhibition of either alga was observed.

Streptomycin. Zuraw et al (1960), reference 13, reported that Streptomycin in concentration up to 10^{-5}M had no effect on Chlorella (71105). Provasoli et al (1951), reference 10, were successful in their studies in obtaining monoalgal, bacteria-free cultures by the use of antibiotics, including streptomycin. A sample of streptomycin sulphate was kindly provided by Chas. Pfizer and Co., Groton, Connecticut.

In our tests, streptomycin at concentration up to 100 mg/L had no effect on Chlorella 71105. Inhibition of the blue-green form occured at levels of 15 to 30 mg/L. However, control of the blue-green alga contaminant in the eight liter culture units with streptomycin at a level of 30 mg/L was not successful. It should be noted that the culture units were operated on a continuous culture basis. Streptomycin added to the reservoir of medium probably lost its activity as time passed. If blue-green contamination occurs in the future, streptomycin will be added directly to the culture vessel.

4. Flask and Test Tube Tests on the Composition of the Medium

Appendix A and Table IX show the ingredients and the concentration of the various salts used in our standard medium for Chlorella 71105. Any detailed study on mineral nutrition of algae would require specially purified salts and pure water.

However, we have routinely used Groton tap water; consequently, tests were conducted to determine the optimum level for some components of the tap water medium. Appendix D&E show the composition of Groton tap water.

In each experiment a single ingredient of the medium was tested. The concentration of this salt was varied while all other components were held at the standard level. After the optimal level for a given salt was determined, that level was then used in subsequent tests.

Tests were carried out in 100 ml test-tubes, incubated in a lighted aquarium (the temperature was maintained at $38^{\circ} \pm .5^{\circ}$C and the light intensity at approximately 2000 foot candles). The carbon dioxide concentration used was 3%±0.5%. Growth was measured by optical density with a Fisher Electro-photometer at 550 mμ. Tests were done in duplicate.

TABLE IX

Mineral Nutrition Studies with Chlorella 71105

	Constituent	Standard Medium	Optimal Levels
Basic Ingredients	$MgSO_4.7H_2O$	5.0 g/L	0.625 g/L
	KH_2PO_4	2.5 g/L	0.078 g/L
	NaCl	2.0 g/L	-
Supplements	Nitrogen (Urea)	400 mg/L	300 mg/L
	Iron (Na_2Fe EDTA)	0.56 mg/L	0.42 mg/L
	Calcium ($CaCl_2$)	8.0 mg/L	-
	Arnon's A-5 Solution	2.0 ml/L	0.5 ml/L

Table IX indicates the level of each ingredient in the standard laboratory medium and the levels found to be optimum for short-term growth. No significant increase in growth was obtained by adding NaCl or $CaCl_2$ at any level up to the standard concentra-

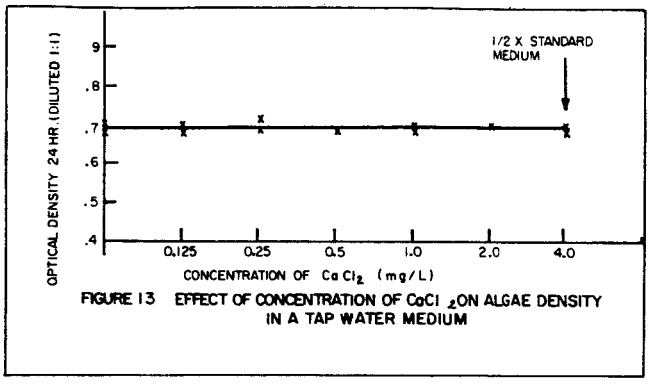

FIGURE 13 EFFECT OF CONCENTRATION OF $CaCl_2$ ON ALGAE DENSITY IN A TAP WATER MEDIUM

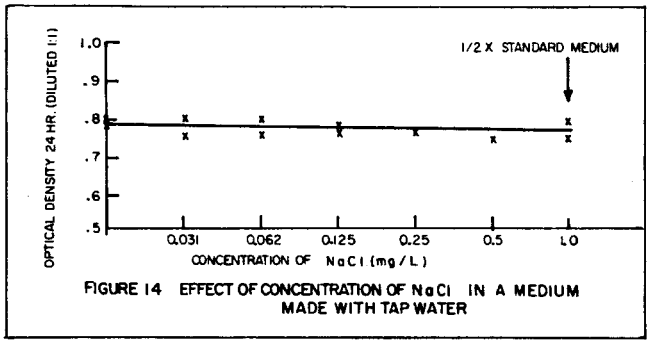

FIGURE 14 EFFECT OF CONCENTRATION OF NaCl IN A MEDIUM MADE WITH TAP WATER

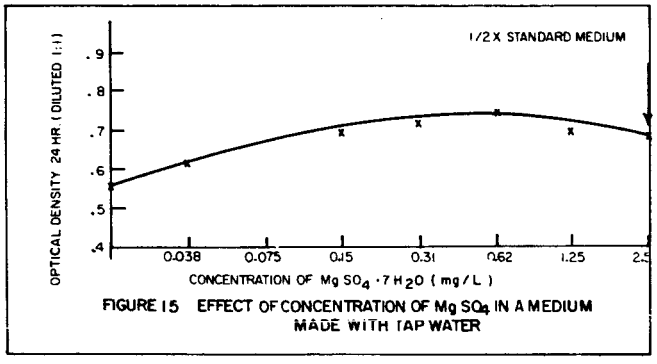

FIGURE 15 EFFECT OF CONCENTRATION OF $MgSO_4$ IN A MEDIUM MADE WITH TAP WATER

tion. Figure 13 and 14 show the results with NaCl and $CaCl_2$. Figure 15 shows the results of the test with $MgSO_4$. A level of 1/8 the standard concentration yielded the highest density. However, cultures grew fairly well in a medium containing no added $MgSO_4$; that is, with only that amount present normally in Groton tap water. Figure 16 shows the results of the tests with KH_2PO_4. Best growth results occurred at a level of 1/32x or at a level of 78 mg/L compared to a level of 2500 mg/L in standard medium.

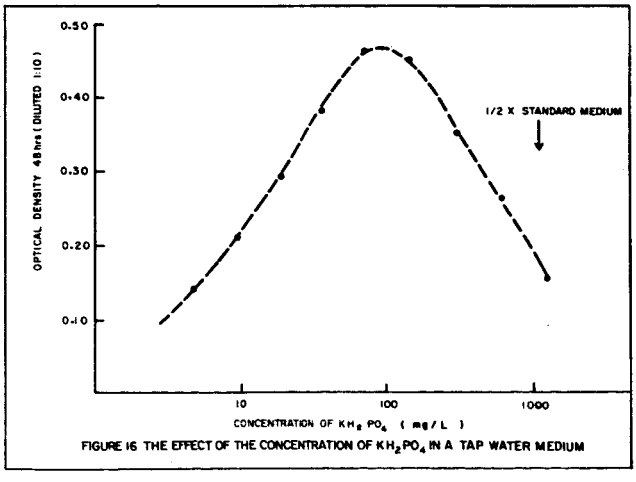

FIGURE 16 THE EFFECT OF THE CONCENTRATION OF KH_2PO_4 IN A TAP WATER MEDIUM

The results of the experiments with urea are shown in Figure 17. Standard medium contains 400 mg/L of urea. No significant increase in density was noted at levels of 100 thru 900 mg/L. A level of 300 mg/L was selected for use in subsequent tests on the composition of the medium.

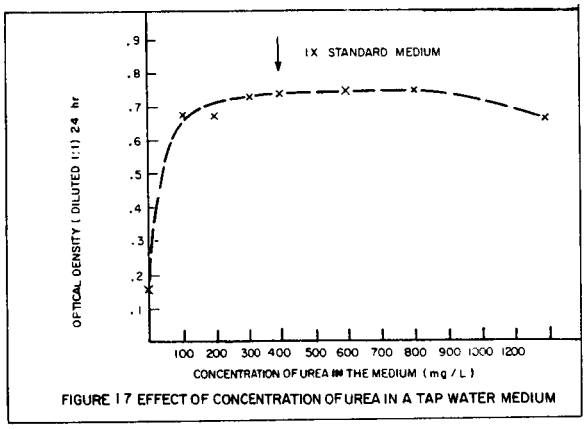

FIGURE 17 EFFECT OF CONCENTRATION OF UREA IN A TAP WATER MEDIUM

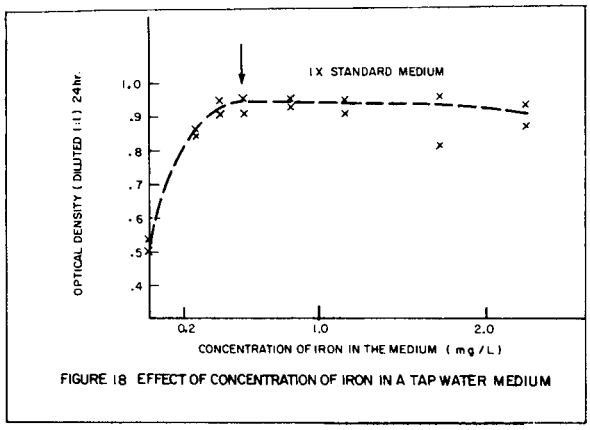

FIGURE 18 EFFECT OF CONCENTRATION OF IRON IN A TAP WATER MEDIUM

The experiment on the concentration of iron is shown in Figure 18. As shown, concentrations of iron from 3/4x to 4x gave no significant change in growth. Standard medium contains 0.56 mg/L of iron. A level of 3/4x was selected for tests on other components of the medium.

Two milliliters of Arnon's A-5 solution (with added sodium vanadate), as prepared in Appendix A, is normally added to one liter of standard medium. Figure 19 shows the results of tests conducted varying the amount of Arnon's A-5 solution. As shown, a level of 0.5 ml/L yielded slightly better results than a level of zero concentration and a level of 2.0 ml/L; therefore, a level of 0.5 ml/L was selected as the optimum level for Arnon's A-5 solution in a tap-water medium. Semi-

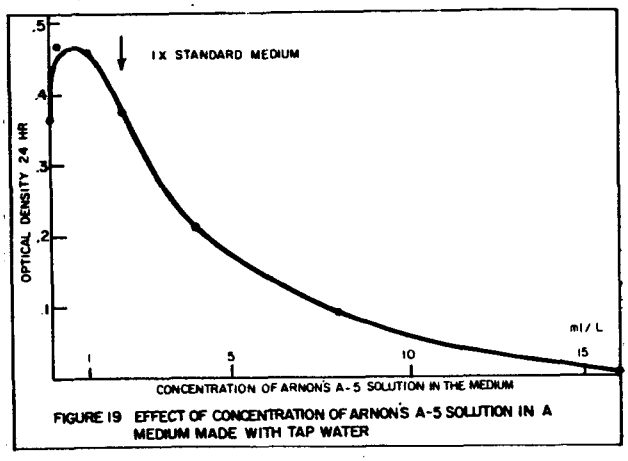

FIGURE 19 EFFECT OF CONCENTRATION OF ARNON'S A-5 SOLUTION IN A MEDIUM MADE WITH TAP WATER

quantitative spectrographic analysis of evaporated residues of Groton tap water indicate that elements in Arnon's A-5 solution are present at levels close to those considered optimal. Some of these elements, especially copper and manganese, are apt to be extremely variable in treated waters. Copper is commonly present at high levels in waters from copper or brass plumbing, but the toxicity of copper and other heavy metals is vitiated by the high levels of the basic salts in the standard medium. We have often omitted Arnon's A-5 solution from the tap water medium with no apparent effects.

To verify the results of the above tests, a 72-hour experiment was conducted comparing the culture densities obtained with a medium containing the ingredients at levels found in standard laboratory medium, and at levels found to be optimum. (The new medium has been designated DL61.) The average optical density attained with standard medium was 0.553 ± 0.001 units (average of seven test tubes, diluted 1:10). The average optical density attained with the DL61 medium was 0.709 ± 0.001 (average of 8 test tubes, diluted 1:10).

It must be noted that the levels of the components of DL61 medium as shown in Table IX are in addition to the levels normally found in Groton tap water. Appendix E lists information concerning the chemical composition of Groton tap water. As shown, Groton tap water contains, sodium, calcium, and chlorides, at levels of 7.0, 8.0, and 8.5 mg/L respectively.

The hardness and alkalinity values given would indicate approximately 20 mg/L of bicarbonate present in Groton tap water.

Standard medium used routinely in these laboratories for growing Chlorella 71105 has a pH of 4.6. To determine the optimal for short-term growth of Chlorella 71105, a series of tests were conducted. In those tests, standard medium was adjusted to pH levels of 6, 7, and 8 with sodium hydroxide solution. After 24 hrs., growth was measured in terms of packed cell volume. The pH was determined initially and at the end of each experiment. Figure 20 shows the results. As indicated, optimum growth occured at a pH of 6.0. Growth in cultures with the medium adjusted to a pH of 6.0 or more grew to twice the density of cultures in the untreated standard medium (pH 4.6). The standard medium contains monobasic potassium phosphate; pH can be conveniently poised by using an appropriate combination of the mono-and dibasic salts. Other aspects of the pH of the medium were studied, the results of these tests are discussed below.

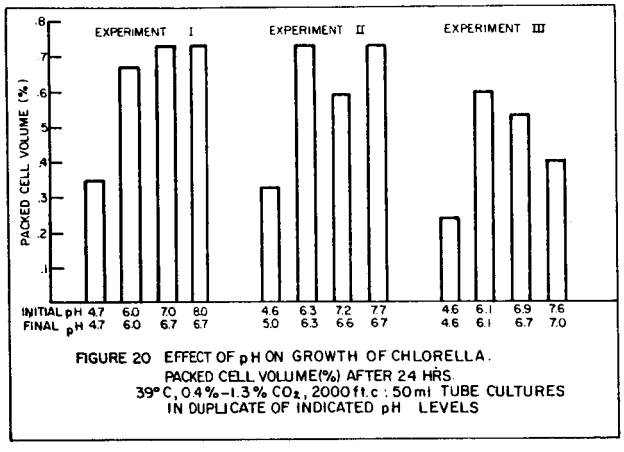

FIGURE 20 EFFECT OF pH ON GROWTH OF CHLORELLA.
PACKED CELL VOLUME(%) AFTER 24 HRS.
39°C, 0.4%-1.3% CO_2, 2000 ft.c : 50 ml TUBE CULTURES
IN DUPLICATE OF INDICATED pH LEVELS

The pH of DL61 medium was determined to be 6.6. In order to determine whether a slightly lower pH would improve the DL61 medium, three sets of five tubes were inoculated and incubated in light for twenty-four hours. The first set contained the DL61 medium (pH 6.6); the second set, DL61 with 0.2 ml/L 1.0N HPO_3 added (pH 5.9); and the third set DL61 with 0.4 ml/L 1.0N HPO_3 added (pH 5.4). The average optical densities after twenty-four hours, 0.45, 0.41, and 0.29; the corresponding pH's were 6.6, 5.9, and 5.4. The difference between

the first two densities is not significant. The pH in the first set remained unchanged after twenty-four hours. In the second set it had increased from 5.9 to 6.0; and in the third set, from 5.4 to 5.7.

It is noteworthy that DL61 medium differs from the standard medium only in the reduction of the levels at which some components are present, and furthermore, in that the pH is slightly higher. The standard medium was originally developed for supporting sustained growth in dense steady-state Chlorella cultures, and it is probably the best that can be devised for this purpose. For short-term growth starting with a very small inoculum, DL61, in Groton tap water, is significantly better and will be used routinely in the future for short-term tests.

V. Conclusion and Recommendations

A. Studies with Supplemented Nutrient Medium

It was confirmed that steady-state algal cultures could be maintained for periods of several weeks by supplementing the nutrient medium with minimal amounts of certain salts. No buildup of autotoxic materials was observed over seventy-two days. Iron and urea were determined to be the most important supplements. Dense cultures were maintained in in the presence of high bacterial populations. When the iron and urea status of steady-state cultures was maintained, growth became limited by lack of calcium and trace elements (Arnon's A-5 solution).

Future studies should include experiments with a continuous recycle of medium; that is, operation which includes a means of harvesting algal cells continuously and returning the spent medium to the culture. This mode of operation will provide a model for the definitive configuration, in which water must be completely recycled and all supplements to the medium must be derived from human wastes.

B. The Factorial Experiment

These experiments confirmed the finding in our earlier report (reference 13) that the higher the ratio of lighted surface to volume of culture, the greater the yield of algae. At the levels of carbon dioxide studied, a 2% concentration supported algae production better than either 1% or 0.03%. Dilution factor (ratio of nutrient feed rate to volume) was shown again to be a significant parameter in the growth of steady-state algae cultures. A factor of 0.07 produced the most algae (per liter of culture). At a value of 0.07, doubling time was 9.9 hr. Highest equilibrium culture density (measured as mg dry weight/liter) was attained at a dilution factor of 0.04. At this level of the factor, a doubling time of 17.3 hr was found. At the highest dilution factor, (0.1), similar specific yields were obtained with cultures of lesser density and a faster generation time (6.9 hr in this instance).

Oxygen production although not directly measured in these experiments can be calculated using the data of Myers (reference 9). He reported that 1 gm of dry algae is equivalent to 1.1 liters of oxygen. Under optimum condition in these experiments, 0.67 gm/hr of algae was produced, which is equivalent to 0.74 liters of oxygen/hr. Assuming that man requires 1.0 SCF of oxygen per hour, the culture vessel with five liters of algae suspension and operating at 1200 watts (240 volts) is equivalent to 0.026 men. The efficiency of light utilization was consequently about five per cent.

It is recommended that future studies include more levels of the ratio: lighted area to culture volume. The continued study of this parameter is important for future designs.

Increased levels of carbon dioxide are also indicated as worthy of future attention, since the highest level used in these studies gave the greatest yields both in terms of equilibrium culture density and algal production. The level of light intensity used was suspected to be suboptimal, but was forced by expediency. Future studies with the eight-liter vessel should be conducted at a higher level of light intensity.

C. Flask and Test Tube Studies

These studies disclosed that standard nutrient could be stored for long periods of time with little change in the urea, nitrogen or pH value. Algae inoculum can also be stored in the refrigerator for long periods of time as either a dilute or a concentrated suspension. Upon re-culturing, cells stored as a dilute cell suspension display a lag period; cells stored as a concentrated suspension show a longer lag.

Streptomycin has no effect on Chlorella 71105 up to concentration of 100,000 µg/L. Control of a blue-green contaminant was achieved by the use of streptomycin.

Tests on the composition of the medium made in Groton tap water indicated that some constituents should be reduced or eliminated for short-term growth studies, optimim levels of each ingredient for short-term growth were determined. The formula of the new tap-water medium (DL61) is given in Table IX.

A pH level of 6.0 was found to be better for growth of Chlorella than the pH of 4.6 in standard laboratory medium.

APPENDICES

APPENDIX A

Standard Laboratory Medium

Basic Medium

Constituent	gms/L
$MgSO_4 \cdot 7H_2O$	5.0
KH_2PO_4	2.5
NaCl	2.0

Dissolved in tap water to make 1 liter

Supplemental Solutions

Element	Source	Final Concentrations of Element in Basic Medium (mg/L)
Nitrogen	Urea	186
Iron	$Na_2FeEDTA$	0.56
Calcium	$CaCl_2$	8.0
Arnon's A-5 Solution*		
B	H_3BO_3	0.99
Mn	$MnCl_2 \cdot 4\ H_2O$	1.0
Zn	$ZnSO_4$	0.18
Cu	$CuSO_4 \cdot 5H_2O$	0.04
Mo	$(NH_4)_6\ Mo_7O_{24} \cdot 4H_2O$	0.003
V	$Na_3VO_4 \cdot 16H_2O$	0.009

*A stock solution in 0.01 N H_2SO_4 is prepared and used at 2 ml/L

APPENDIX B

Procedure for Calculating Equilibrium Density and Algae Production

1. 40 ml of the algal culture was removed from the unit with a volumetric pipette.

2. This suspension was centrifuged and the resultant supernatant fluid discarded.

3. The packed cells were then washed in de-ionized water three times.

4. After the third washing, the volume was adjusted to 20 ml. (This doubled the original concentration of cells.)

5. 3 ml of suspension were centrifuged in duplicate in graduated test tubes. The average of the two was the value noted for the packed cell volume.

6. 10 ml of the suspension prepared in 4 above were distributed into previously dried and weighed aluminum weighing dishes; again duplicate samples were run.

7. The algae were then dried for 24 hrs at 105°C, cooled, and then weighed. Weight divided by 20 equals suspension density (mg/ml).

8. Suspension density times flow rate in ml/hr divided by 1000 was taken as yield in grams of dry algae per hour.

9. Specific yield (grams of dry algae per liter per hour) was obtained by dividing yield by culture volume.

APPENDIX C

Light Measurements of the Eight-liter Units

1. Light measurements were taken using a G.E. light meter and a Weston foot-candle meter, Model 614, with neutral density filters of 1.0 and 0.9.

2. Measurements taken using the G.E. meter were taken at points 1 inch from either end of the annulus and at the center on the top portion of the annuli. Readings were also taken at points 1.5 inches on either side of these points. All values were averaged.

3. Measurements taken using the Weston foot-candle meter were taken at 1 inch from either end and at the center of the top of the annuli.

4. Results are tabulated below:

Foot Candles*

		Unit #1	Unit #2
Lamp Voltage	G.E. Meter	5900	5700
240 Volts	Weston Meter	6240	7150
	Mean	6070	6420

*Readings taken upon completion of experimentation.

APPENDIX D

Semi-quantitative Spectrographic Analysis of Evaporation Residue from Groton Tap Water (1)

Greater than 10%	1.0% to 10%	0.1% to 1.0%	0.01% to 0.1%	0.001% to 0.01%	Less than 0.001%	Less than 0.0005%
Calcium	Silicon	Aluminum	Barium	Indium	Antimony	Bismuth
Sodium	Magnesium	Boron	Copper	Lead	Arsenic	Iridium
		Iron	Manganese	Molybdenum	Chromium	Silver
			Zirconium	Nickel	Cobalt	Tin
				Potassium	Lithium	
				Strontium	Phosphorus	
				Titanium	Rhodium	
				Vanadium		
				Zinc		

(1) Reference 6

APPENDIX E

Chemical Analysis of Groton Tap Water *

1. pH----------7.2 (average 6 days, June 1961)
2. Chloride----------8.5 mg/L (average 6 days, June 1961)
3. Hardness (as $CaCO_3$)---------33 mg/L (average 6 days, June 1961)
4. Copper----------0.007 mg/L (average 6 days, June 1961)
5. Organics (Extracted in CCl_4)0.13 mg/L (average 6 days, June 1961)
6. Conductivity----------85.5 µmhos/cm^3 @ 25°C (average 6 days, June 1961)
7. Sodium----------7.0 mg/L (analysis 1961)
8. Calcium----------8.0 mg/L (analysis 1961)
9. Potassium----------1.0 mg/L (analysis 1961)
10. Total Solids----------56.2 mg/L (analysis 1956)
11. Dissolved Solids----------55.0 mg/L (analysis 1956)
12. Suspended Solids----------1.2 mg/L (analysis 1956)
13. Alkalinity (phenolphthalein)0 (analysis 1956)
14. Alkalinity (methyl orange)--8.0 mg/L (analysis 1956)
15. Chlorine Residual----------0.75-0.90 mg/L (leaving treatment plant)

*Items 1, 3, and 15 were obtained from the Groton, Connecticut Water Department. All other information was obtained from the Analytical Chemistry Section, Technical Services Division, Electric Boat.

VII

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